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STIMULATORY EFFECTS OF CALCIUM CARBONATE ON BUTANOL PRODUCTION BY SOLVENTOGENIC *Clostridium* species

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ABSTRACT
Solventogenic *Clostridium* species are motile Gram positive anaerobic bacteria used for the fermentative production of acetone-butanol-ethanol (ABE). The addition of $CaCO_3$ to semi-defined P2 medium was investigated to evaluate its impact on growth, amelioration of butanol toxicity, glucose utilization, and ABE production by solventogenic *Clostridium* species. Two *Clostridium* species commonly used in industrial production of fermentative-derived ABE were used in the study. Under culture conditions of the present study, $CaCO_3$ enhanced growth of and total ABE production by solventogenic *Clostridium* species (*C. acetobutylicum* ATCC 824 and *C. beijerinckii* 260 produced more than 24 and 22 g/L ABE, respectively) by 31% to 46%. Dramatically increased growth of solventogenic *Clostridium* species and fermentation rate resulted in greater ABE concentration and productivity for treatments containing $CaCO_3$ relative to the control with no $CaCO_3$ addition. The addition of $CaCO_3$ into P2 medium, in addition, increased butanol tolerance by more than 40% indicating that elevated levels of ABE produced by solventogenic *Clostridium* species may have occurred due to enhanced tolerance of butanol in the presence of $CaCO_3$.

KEYWORDS: Butanol, acetone, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, Calcium carbonate, biofuel

INTRODUCTION

Interest in biofuel production has grown recently due to continuous depletion of worldwide oil deposits, awareness in greenhouse gas (GHG) emissions during combustion of fossil fuels, and potential impacts of this activity on global warming. Ethanol is currently the most important renewable liquid biofuel, but it has problems ranging from lesser energy content than gasoline, blending limitations with gasoline, potential for corrosion of pipes, and inability to be transported using existing pipeline infrastructure (Ezeji and Blaschek, 2010). The acetone butanol ethanol (ABE) fermentation process is of particular interest for the production of fuels and chemicals from renewable resources because butanol has superior attributes over other fermentation-derived fuels, including ethanol (Ezeji and Blaschek, 2007). Butanol, currently manufactured with petroleum feedstock, has a lower vapor pressure than ethanol which makes it less flammable, safer to transport, and safer to use in combustion engines than ethanol (Dürre, 2008). Major challenges with ABE fermentation are limitations such as low ABE concentration, yield, and productivity due to butanol toxicity to microbial cells (Ezeji *et al*., 2003; 2004). Currently, butanol is not used as a biofuel because of cost. However, David Ramsey, Environmental Energy Inc., The Ohio State University, drove an unmodified 1992 Buick 10,000 miles across the USA using only butanol as a fuel (Ezeji and Blaschek, 2007).

With the history of bio-butanol production by the natural solvent-producing *Clostridium* species such as *Clostridium acetobutylicum* 824, and *Clostridium beijerinckii* 260, there are some clear inherent advantages in improving and using these native clostridial systems for producing bio-butanol (Ezeji *et al*., 2010). The ABE concentration and productivity in a typical batch fermentation by solventogenic *Clostridium* species can be increased to values between 13 to 18 g/L and 0.2 to 0.3 g/L/h, respectively (Ezeji and Blaschek, 2008). Modest increases in ABE titer and productivity can be achieved by incorporating acetate into fermentation medium (Chen and Blaschek, 1999; Gu *et al*., 2009). Studies focused on butanol removal from the fermentation broth using distillation showed that, as the concentration of butanol increased from 10 g/L to 40 g/L, ratio of oil to

fuel used for 100% recovery of butanol decreased from 1.5 t/t to 0.25 t/t (Philips and Humphrey, 1983). This suggests that significant energy savings can be achieved if the concentration of butanol in the fermentation beer is increased.

ABE production is by biphasic fermentation process in which acetic and butyric acid are produced inside the microbial cells with concomitant release into the environment during the acidogenic phase followed by re-assimilation of the acid into the cells and conversion into ABE (solventogenic phase) (Ezeji *et al*., 2010). It is noteworthy that excessive accumulation of undissociated acetic and butyric acid in the bioreactor during ABE fermentation is detrimental to nutrient uptake by and growth of solventogenic *Clostridium* species (Herrero *et al*., 1985).

The objective of this study was to examine the effect of $CaCO_3$ addition in chemically semi-defined media on growth and ABE production by solventogenic *Clostridium* species. Although the two microorganisms used for the study have significant differences in both genome and physiology (Nolling *et al*., 2001), we investigated whether the effect of $CaCO_3$ is species and strain specific or non-selective in action.

MATERIALS AND METHODS

Microorganism, culture maintenance and inoculums development

Microorganisms used for this study were *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* 260. *C. acetobutylicum* ATCC 824 was obtained from America Type Culture Collection in Manassas, Virginia, USA, while *C. beijerinckii* 260 was obtained from Professor David Jones. Laboratory stocks of *C. beijerinckii* 260 and *C. acetobutylicum* ATCC 824 were routinely maintained as spore suspensions in sterile double distilled water at 4 °C. *C. acetobutylicum* ATCC 824 spores (200 ml) was heat shocked for 10 min at 75 °C, while *C. beijerinckii* 260 was heat shocked for 3 min at 65 °C followed by cooling on ice. The heat shocked spores were inoculated into 10 mL anoxic pre-sterilized tryptone–glucose–yeast extract (TGY) medium (Ezeji *et al*., 2003, 2004) and incubated anaerobically for 12 to 14 h at 35 ± 1 °C. *C. beijerinckii* 260 spores may take up to 18 h before any visible growth can be seen. This was followed by transferring 8 mL of actively growing culture (12–14 h old) to 92 mL anoxic pre-sterilized tryptone–glucose–yeast extract (TGY) medium. To create anaerobic conditions and prepare anoxic medium, loosely capped bottles with sterilized TGY medium were kept in the anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Michigan) with modified atmosphere of 82% N_2, 15% CO_2, and 3% H_2 for 24 h to facilitate exchange of gases between TGY medium and gases in anaerobic chamber. Cells were grown anaerobically at 35 °C for 4 to 5 h during which the optical density of cells at 540 nm λ attained 0.9-1.1 (pre-culture).

Effect of calcium carbonate on *Clostridium* species growth and ABE production

Various concentrations (2-10 g/L) of $CaCO_3$ were added into 250 mL Pyrex screw capped bottles containing 60 g/L glucose and 1 g/L yeast extract. The mixture was sterilized at 121 °C for 15 min. On cooling to 37 °C, the bottles were transferred into an anaerobic chamber at 35 °C for 24 h for anaerobiosis. This was followed by addition of filter-sterilized P2 stock solutions and inoculation with pre-culture (*C. acetobutylicum* ATCC 824 or *C. beijerinckii* 260) as described previously (Ezeji *et al*., 2003). Samples (5 mL) were collected every 12 h for cell concentration, ABE, glucose and acid analysis. Unless otherwise stated, all fermentations were conducted in triplicate with the cultures grown with no agitation at 35°C, and no special measures taken to control pH.

Effect of calcium carbonate on protein expression in *Clostridium* species

To evaluate whether addition of $CaCO_3$ into growth medium enhanced protein expression in *Clostridium* species, *C. beijerinckii* cells were harvested from P2 medium (control) and P2 medium plus $CaCO_3$ (treatment) fermentation and used for analysis. To eliminate bias in protein concentration due to cell concentration, control and treatment cells were adjusted to same optical density at 540 nm wave length using DU800 spectrophotometer. *C. beijerinckii* cells were lysed with TissueLyser LT (Qiagen Inc., Valencia, CA), centrifuged at 14,000 x *g* for 10min and supernatant was used as crude protein. Aliquots of the crude protein were loaded onto SDS gel followed by SDS-PAGE analysis following standard procedure.

Analytical procedures

Growth of *C. acetobutylicum* ATCC 824 or *C. beijerinckii* 260 was estimated by measuring OD_{540} using a DU800 spectrophotometer (Beckman Coulter Inc., Brea, CA). ABE and acid (acetic & butyric) concentrations were measured using a 7890A Agilent Technologies gas chromatograph (Agilent Technologies Inc., Wilmington, DE) equipped with a flame ionization detector (FID) and 30 m (length) x 320 µm (internal diameter) x 0.50 µm (HP-Innowax film) J x W 19091N-213 capillary column. ABE productivity, or rate of production, was calculated as total ABE produced (g/L culture volume) divided by fermentation time (h). Glucose concentration was determined using a hexokinase and glucose-6-phosphate dehydrogenase coupled enzymatic assay (Ezeji and Bahl, 2006). Glucose utilization rate was calculated as total glucose utilized by solventogenic *Clostridium* species (g/L culture volume) divided by fermentation time (h). Yield was defined as total grams of ABE produced per total grams of glucose utilized.

To evaluate whether addition of $CaCO_3$ into growth medium reduced the toxic effect of butanol on *Clostridium* species, *C. acetobutylicum* 824 and *C. beijerinckii* 260 were grown in P2 medium containing 0-20 g/L butanol and incubated in an anaerobic chamber at 35 °C for 24 h as described above.

RESULTS AND DISCUSSION

Effect of $CaCO_3$ on growth, pH and acetic-butyric acid production by solventogenic *Clostridium* species

To evaluate impact of $CaCO_3$ on growth of solventogenic *Clostridium* species, batch fermentations were conducted with P2 medium containing different concentrations of $CaCO_3$. The growth of *C. acetobutylicum* was enhanced in the presence of different concentrations of $CaCO_3$ (Fig. 1) reaching average maximum cell density of 9.31 (Table 1A) which resulted in about a 2-fold increase in growth when compared to the control.

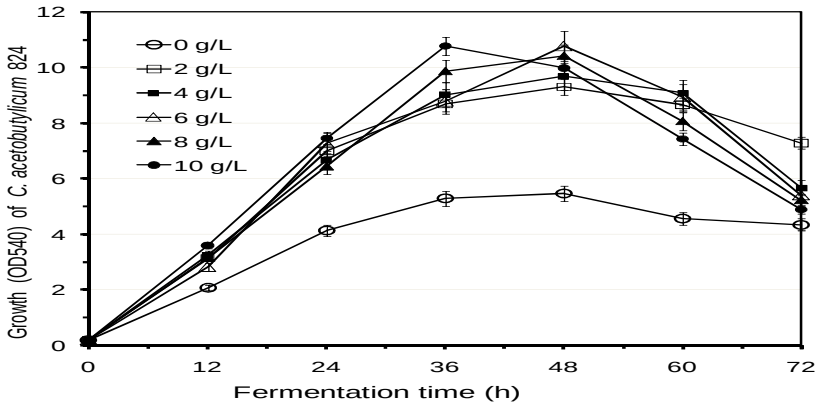


Figure 1: Optical density of *C. acetobutylicum* 824 (measure of cell growth) obtained during batch ABE fermentation in P2 medium containing different concentrations (0-10 g/L) of $CaCO_3$. The data represent the averages of ≥ 3 fermentations.

Similar results were obtained with *C. beijerinckii* 260 (Table 1B) where average optical density reached 8.98. With the exception of the 2 g/L $CaCO_3$ treatment, no significant difference in glucose utilization among other (4 to 10 g/L) $CaCO_3$ treatments (data not shown) was observed. Hence optimal concentration of $CaCO_3$ for the growth of solventogenic *Clostridium* species is 4 g/L. Because $CaCO_3$ enhanced solventogenic *Clostridium* species cell growth to a great extent, we determined the effect of $CaCO_3$ on the rate of glucose utilization. We compared results obtained from fermentations containing no $CaCO_3$ supplementation (control) with fermentations containing 4 g/L $CaCO_3$ (Table 1A and 1B). Addition of 4 g/L $CaCO_3$ to fermentation medium resulted in a glucose utilization rate of 1.2 g/Lh compared to the control whose values ranged from 0.6 to 0.8 g/Lh glucose (Table 1A and 1B), a 50 – 100% increase. ABE fermentation under conditions of excess glucose (60 g/L) is typical because excess sugar in the fermentation medium is essential for maintenance of solvent production (Jones and Woods, 1986). At the end of fermentation, however, about 10-20 g/L glucose is left in the bioreactor due to inability of *Clostridium* species to use all substrates in the presence of relatively high

butanol concentrations (Grupe and Gottschalk, 1992; Qureshi and Blaschek, 2000; Ezeji *et al*., 2007a, 2007b). In the presence of $CaCO_3$, residual concentration of glucose in the bioreactor was zero following 48 h fermentation by *C. beijerinckii* (Table 1B). Past economic analysis demonstrated that the fermentation substrate was one of the most important factors that influenced the cost of fermentation-derived butanol production (Qureshi and Blaschek, 2000).

Table 1A: Total sugar utilized, ABE produced, ABE yield and productivity obtained during batch ABE fermentation by *C. acetobutylicum* ATCC 824 in the presence 4 g/L $CaCO_3$. No $CaCO_3$ was present in the control.

Parameters (Max. values)	Control	$CaCO_3$ treatment
Acetone (g/L)	3.66 ± 0.44	7.62 ± 0.26
Ethanol (g/L)	1.30 ± 0.04	1.16 ± 0.20
Butanol (g/L)	11.43 ± 1.00	14.78 ± 0.32
Total ABE (g/L)	16.39 ± 1.45	24.01 ± 0.73
Initial glucose (g/L)	60.17 ± 0.53	59.28 ± 0.83
Final glucose (g/L)	16.02 ± 1.23	1.61± 0.63
Cell density (540 nm)	5.47 ± 0.09	9.31 ± 0.21
Fermentation time (h)	72	48
Total glucose utilized (g)	44.15 ± 0.71	57.68 ± 0.20
Glucose utilization rate (g/Lh)	0.61 ± 0.01	1.2 ± 0.0
ABE Yield (g/g)	0.37 ± 0.03	0.42 ± 0.01
ABE Productivity (g/L/h)	0.23 ± 0.02	0.50 ± 0.04

Table 1B: Total sugar utilized, ABE produced, ABE yield and productivity obtained during batch ABE fermentation by *C. beijerinckii* 260 in the presence 4 g/L $CaCO_3$. No $CaCO_3$ was present in the control.

Parameters (Max. values)	Control	$CaCO_3$ treatment
Acetone (g/L)	5.06 ± 0.14	7.40 ± 0.33
Ethanol (g/L)	0.88 ± 0.04	1.39 ± 0.04
Butanol (g/L)	11.38 ± 0.24	13.89 ± 0.14
Total ABE (g/L)	17.31 ± 0.15	22.68 ± 0.44
Initial glucose (g/L)	59.07 ± 1.8	58.63 ± 0.6
Final glucose (g/L)	10.6 ± 0.34	0
Cell density (540 nm)	4.89 ± 0.34	8.98 ± 0.31
Fermentation time (h)	60	48
Total glucose utilized (g)	48.47 ± 1.02	58.63 ± 0.6
Glucose utilization rate (g/Lh)	0.81 ± 0.02	1.22 ± 0.0
ABE Yield (g/g)	0.36 ± 0.01	0.40 ± 0.004
ABE Productivity (g/L/h)	0.29 ± 0.003	0.47 ± 0.01

Complete utilization of glucose by *C. beijerinckii* 260 (Table 1B) in batch fermentation has the potential to improve the economics of fermentation-derived butanol production and effluent disposal.

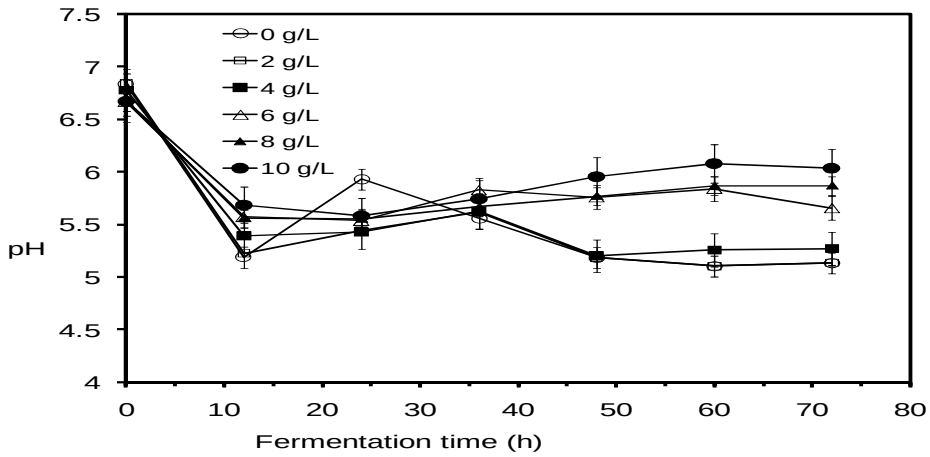
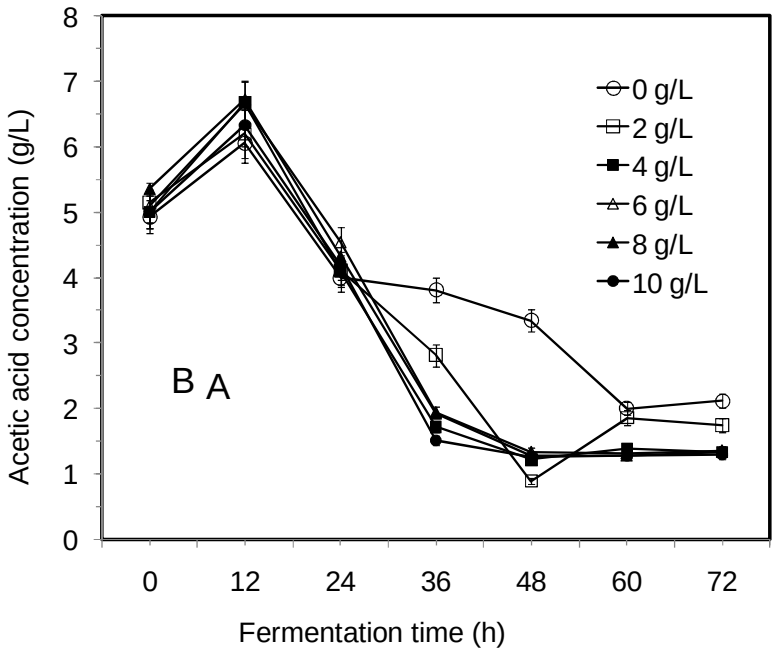


Figure 2: The pH profile obtained during batch ABE fermentation by *C. acetobutylicum* 824 in P2 medium containing different concentrations of $CaCO_3$. The data represent the averages of ≥ 3 fermentations.

Furthermore, during growth of solventogenic *C. acetobutylicum*, low pH < 5.5 (Fig. 2) and accumulation of acetic-butyric acid (Fig. 3A and 3B) in the bioreactor are necessary for the initiation of ABE production (Ezeji *et al*., 2010). As a result, the effect of $CaCO_3$ upon pH and acid production were measured. The elevated concentrations of total acid measured at 0 h fermentation originated from P2 medium component (ammonium acetate) and in all fermentations, solventogenesis was initiated at the fermentation time of 12 h (Fig. 2, Fig. 3A-B). The pH decreased sharply following 12 h fermentation and the subsequent decrease has no linear relationship with total acids produced during the course of fermentation (Fig. 2, Fig. 3A-B).

This observation agrees with previous related reports (Andersch and Gottschalk, 1982; Bahl *et al*., 1982; Grupe and Gottschalk, 1992) which demonstrated this pattern of pH and total acid fluctuation during growth of solventogenic *Clostridium* species and initiation of solventogenesis. Thorough examination of Figures 2A and 2B shows that greater concentration of acetic-butyric acid was produced by fermentations with $CaCO_3$ than the control with no $CaCO_3$. This greater concentration of acetic-butyric acid, however, did not result in a lower pH than the control due to modulation of dissociated and undissociated acetic-butyric acid by $CaCO_3$.



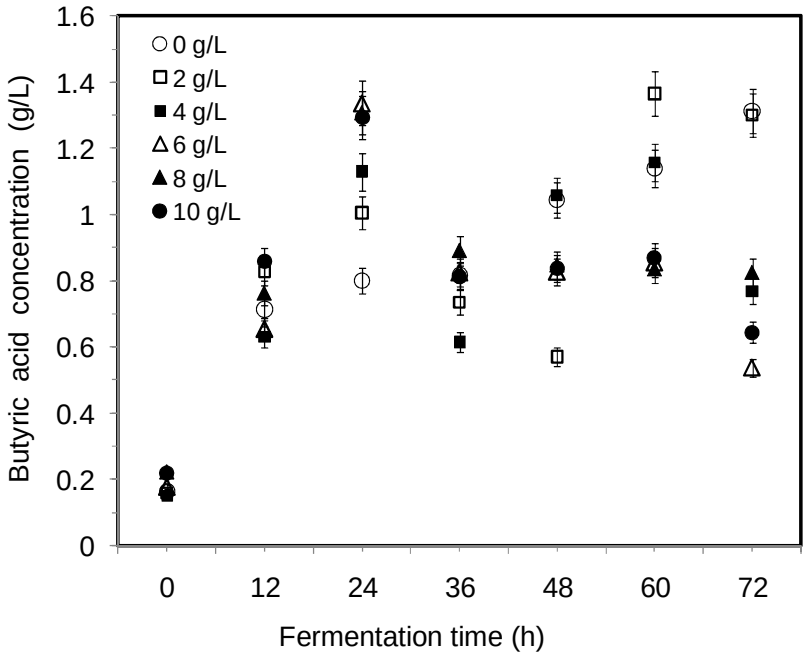


Figure 3: Acetic acid (A) and butyric acid (B) production profiles obtained during batch ABE fermentation by *C. acetobutylicum* 824 in P2 medium containing different concentrations of $CaCO_3$. The data represent the averages of ≥ 3 fermentations.

Poorly buffered fermentation medium will lead to excessive accumulation of undissociated acetic and butyric acid, which is detrimental to nutrient uptake, cell growth, and ABE production (Ezeji *et al*., 2010). Bryant and Blaschek (1988) reported previously an increased buffering capacity of the fermentation medium led to enhanced butanol production although maximum butanol concentration achieved in their investigation was 10 g/L compared to 14.78 g/L butanol (Table 1A) and 13.89 (Table 1B), a 39 – 48% increase, in the present study. Modulation of equilibrium between dissociated and undissociated acetic-butyric acid in fermentation medium, in addition, has been thought to be vital for optimal butanol production by solventogenic *Clostridium* species (Ezeji *et al*., 2010). Excessive buffering will also have a negative impact on butanol production but maintenance of optimal buffering condition in the bioreactor during ABE fermentation for optimal butanol production is crucial.

Effect of $CaCO_3$ on butanol production, productivity and yield by solventogenic *Clostridium* species

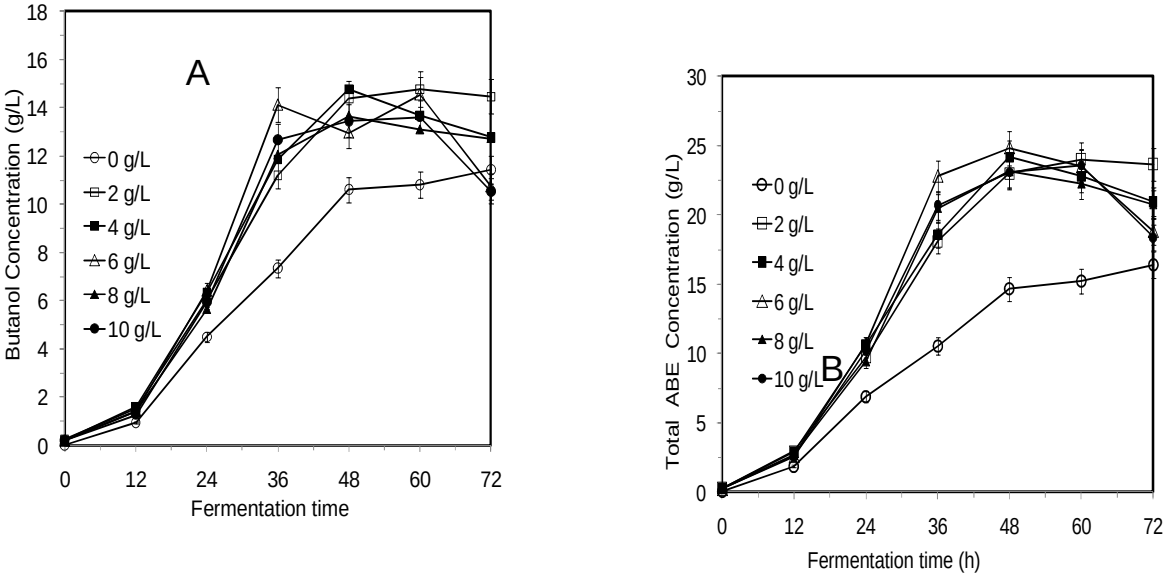


Figure 4: Total butanol (A) and ABE (B) production profiles obtained during batch ABE fermentation by *C. acetobutylicum* 824 in P2 medium containing different concentrations of

Having ascertained that impact of $CaCO_3$ on solventogenic *Clostridium* species growth was not specific for *C. acetobutylicum*, rather exerts same effect on other solventogenic *Clostridium* species, *C. beijerinckii*, the ABE production characteristics of *C. acetobutylicum* 824 and *C. beijerinckii* 260 grown in P2 medium containing various concentrations (2g/L-10g/L) of $CaCO_3$ was conducted and results are presented in Figure 4A-B and Table 1A (*C. acetobutylicum* 824) and Table 1B (*C. beijerinckii* 260). Control fermentations of *C. acetobutylicum* and *C. beijerinckii* without $CaCO_3$ produced maximum butanol concentration of 11.43 g/L and 11.38 g/L, respectively (Tables 1A-B). Increasing $CaCO_3$ concentration in the fermentation medium resulted in enhanced butanol and total ABE production by *C. acetobutylicum* but increase in butanol and ABE production peaked at 4 g/L $CaCO_3$ (Fig. 4A-B). Similar results were obtained with *C. beijerinckii* (Figure 5). Hence optimal concentration of $CaCO_3$ for butanol production is 4 g/L.

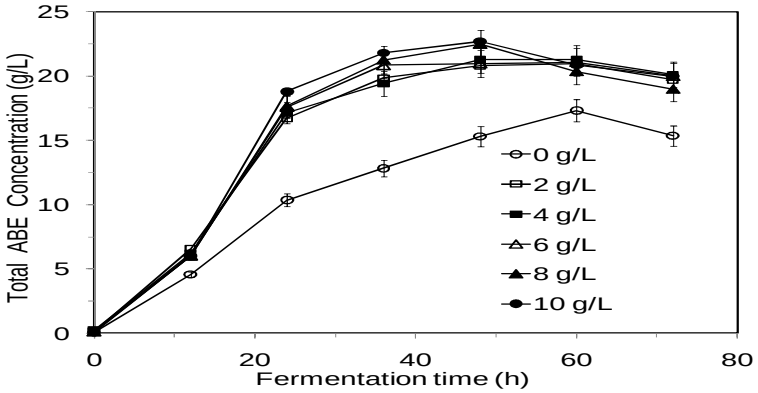


Figure 5: Total ABE production profile obtained during batch ABE fermentation by *C. beijerinckii* 260 in P2 medium containing different concentrations of $CaCO_3$. The data represent the averages of ≥ 3 fermentations.

When total ABE produced by $CaCO_3$ treatment was compared to respective controls of solventogenic *Clostridium* species, *C. acetobutylicum* and *C. beijerinckii* achieved a 46% and 31% increase in ABE production, respectively. ABE productivity by *C. acetobutylicum* and *C. beijerinckii* 8052 was increased by 117% and 62%, respectively (Table 1A-B). ABE yield in solventogenic *Clostridium* species was increased as well by $CaCO_3$ ranging from 11% to 13% (Table 1A-B). Incorporation of $CaCO_3$ into fermentation medium, in addition, resulted in a 24 h reduction in fermentation time (48 h) and more importantly in efficient utilization of glucose (Tables 1A-B).

$CaCO_3$ and total protein expression in solventogenic *Clostridium* species

As part of initial efforts to unravel the molecular basis for the dramatic increase in ABE production and glucose utilization by solventogenic *Clostridium* species that is induced by $CaCO_3$, we inquired if there was up-regulation of select proteins. By using OD_{540} values, we first normalized the amount of cells in the control and $CaCO_3$ treatment (~ 1.8 mg dry weight cells) and ensured that any increase in protein levels detected would be directly attributable to increased protein synthesis and not cell growth. The total crude lysates obtained from these cells were then subjected to SDS-PAGE analysis (Ezeji and Blaschek, 2006). Indeed, in *C. beijerinckii* grown in P2 medium with $CaCO_3$, there is a noticeable increase in the abundance of several proteins; this increase is both proportional to the amount of $CaCO_3$ in the medium and the time of fermentation (Fig. 6). While it is conceivable that these up-regulated proteins might be involved in glucose uptake and utilization, such an idea will await confirmation by our future proteomic investigations.

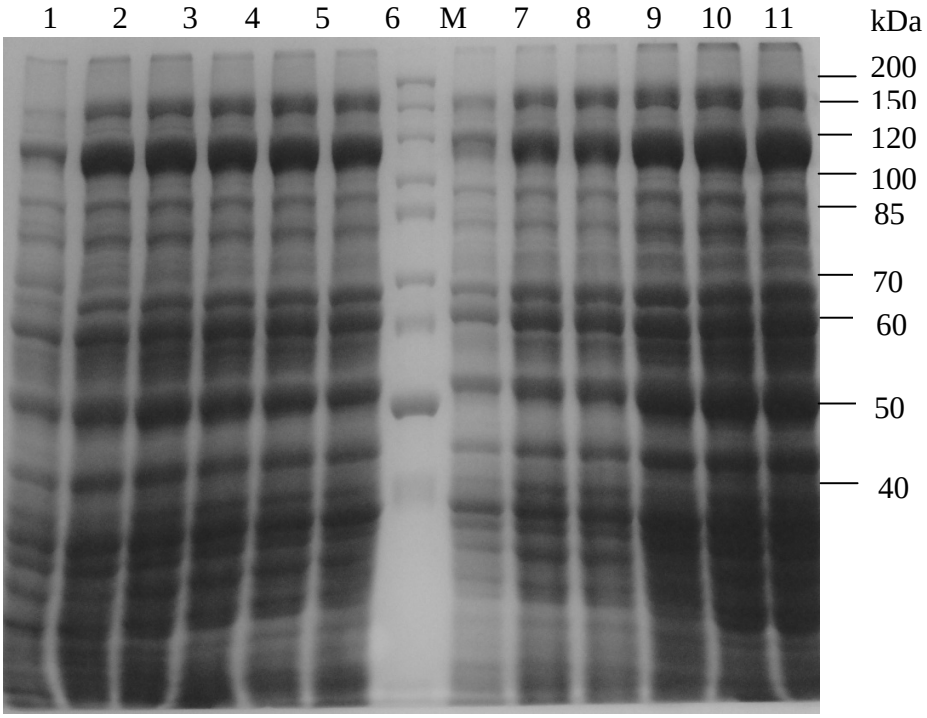


Figure 6: SDS-PAGE of total proteins of *C. beijerinckii* cells harvested at fermentation time of 36 h (line 1-6) and 48 h (line 7-12) during batch ABE fermentation in P2 medium containing different concentrations of $CaCO_3$. Lines 1-6 are samples from P2 medium, P2 medium with 2, 4, 6, 8 and 10 g/L $CaCO_3$ treatments (fermentation time, 36 h). Lines 7 to 12 are *C. beijerinckii* 8052 cells from P2 medium, P2 medium with 2, 4, 6, 8 and 10 g/L $CaCO_3$ treatments (fermentation time, 48 h). Line M is protein ladder standard.

Table 2: Amelioration of butanol toxicity to solventogenic *Clostridium* species (*C. acetobutylicum* 824, and *C. beijerinckii* 260) by $CaCO_3$. Both species had same growth characteristics during growth in media supplemented with different concentrations of butanol.

Treatments	Growth				
	0 g/L butanol	10 g/L butanol	12 g/L butanol	15 g/L butanol	20 g/L butanol
Control (P2 Medium)	++++++++	++++	+	-	-
P2 Medium + 4 g/L $CaCO_3$	++++++++	+++++++	++++	+	-

+: growth; -: no growth; Number of + indicates intensity of growth.

Effect of butanol on growth of solventogenic *Clostridium* species

To evaluate performance of solventogenic *Clostridium* species when fermentation medium is supplemented with butanol, batch fermentations were conducted with P2 medium containing different concentrations of butanol (0-20 g/L) or P2 medium containing 4 g/L $CaCO_3$ and different concentrations of butanol (0-20 g/L). Solventogenic *Clostridium* species grew slowly in all P2 media containing 4 g/L $CaCO_3$ except in treatment containing 20 g/L butanol, unlike P2 medium with no $CaCO_3$ where *Clostridium* species barely grew in 12 g/L butanol treatment (Table 2). This demonstrates that the presence of $CaCO_3$ in the fermentation medium increased the threshold for butanol toxicity to *Clostridium* species. However, growth of solventogenic *Clostridium* species was prematurely terminated in treatments containing ≥ 15 g/L butanol following 8 h of post inoculation to the fermentation medium.

CONCLUSION

We have shown that $CaCO_3$ enhanced growth of solventogenic *Clostridium* species and butanol production. In addition, it appears $CaCO_3$ reduced the impact of butanol toxicity to solventogenic *Clostridium* species which lead to greater accumulation of butanol in the fermentation broth during ABE fermentation. Effect of $CaCO_3$ on solventogenic *Clostridium* species is non-selective in action and this finding may be applied to other microorganisms that undergo biphasic fermentation. Elevated protein expressions in solventogenic *Clostridium* species during fermentations in the presence of $CaCO_3$ are associated with enhanced growth and ABE production; the identity of these proteins and their mechanism of action remain to be deciphered.

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